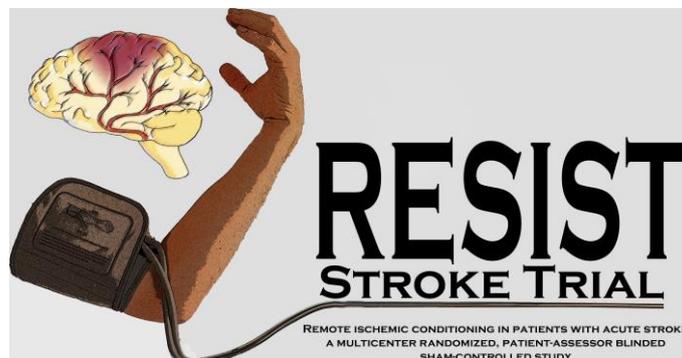


Clinical Investigation Plan



Remote ischemic conditioning in patients with acute stroke: a multicenter randomized, patient-assessor blinded, sham-controlled study.

A multicenter, prospective, randomized, patient-assessor blinded, sham controlled study investigating whether remote ischemic conditioning (RIC) can improve recovery.

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| <u>Project acronym</u> | <u>RESIST stroke trial</u> |
| | REmote iSchemic conditioning In patients with acute STroke: a multicenter randomized, patient-assessor blinded, sham controlled study examining the effect on recovery |
| <u>Clinical investigation plan</u> | <u>Version 8.0, 29th of November 2018</u> |
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RESIST trial is conducted according to the clinical investigation plan and current Danish legislation

The Clinical Investigation Plan will be approved and signed by all investigators before study start.

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1. Synopsis

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| <p>Name of the Sponsor/principal investigator Grethe Andersen, Senior Consultant, MD, DMSc, Professor of Neurology, Department of Neurology, Aarhus University Hospital, DK-8000, Denmark</p> |
| <p>Name of investigational medical device: “Stroke RIC” & “Sham RIC” The investigational devices is developed in collaboration with the Faculty of Biomedical Engineering, Aarhus University, 8200-DK, Aarhus N, Denmark; Seagull Aps, 4160-DK, Herlufmagle, Denmark, and the Department of Neurology, Aarhus University Hospital, 8000-DK, Aarhus C, Denmark.</p> |
| <p>Title of study: RESIST Remote ischemic conditioning in patients with acute stroke: a multicenter randomized, patient-assessor blinded, sham-controlled study, examining the effect on recovery.</p> |
| <p>Trial Management Groups (TMG): Principal investigator/Sponsor (Aarhus University Hospital): Grethe Andersen, Senior Consultant, MD, DMSc, Professor of Neurology, Department of Neurology, Aarhus University Hospital, Nørrebrogade 44, 8000 Aarhus C, Denmark</p> <p>Study Coordinator/Investigator Rolf Ankerlund Blauenfeldt, MD, PhD student. Department of Neurology, Aarhus University Hospital, Nørrebrogade 44, 8000 Aarhus C, Denmark</p> <p>Trial Steering Committee (TMC): Grethe Andersen (Aarhus University Hospital), Rolf Ankerlund Blauenfeldt (Aarhus University Hospital), Niels Hjort (Aarhus University Hospital), Hans Erik Bøtker (Aarhus University Hospital), David C. Hess (Medical College of Georgia, USA), Hans Kirkegaard (Aarhus University Hospital). Rikke Bay Thomsen (Aarhus University Hospital), Birgitte Forsom Sandal (Regional Hospital West Jutland, Holstebro) and Marc Fisher (Beth Israel Deaconess Medical Center, Harvard Medical School, USA).</p> <p>Data Monitoring Committee (and Trial Safety Committee): The DMSC comprises of: Jesper Petersson (Skåne University Hospital, Sweden) – chair. Jan Brink Valentin (Aalborg University Hospital, Denmark) Thomas Christensen (Nordsjællands Hospital, Denmark)</p> <p>Trial Endpoints Validation Committee: EVC comprises of Independent senior consultants in Neurology and Cardiology</p> <p>Trial monitoring: During the entire study period and at all participating centres the regional GCP unit will perform quality assurance control including source data verification (GCP Unit Aarhus/Aalborg, ID 2017-718)</p> |
| <p>Study centers: Department of Neurology, Aarhus University Hospital, DK-8000 Aarhus C, Denmark Department of Neurology, Holstebro Hospital, DK-7500 Holstebro, Denmark</p> |

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|--|
| <p>Department of Neurology, Odense University Hospital, DK-5000 Odense, Denmark (Future study center, 2019 or 2020)</p> <p>Department of Neurology, Aalborg University Hospital, DK-9000, Aalborg, Denmark (Future study center, 2019 or 2020)</p> <p>Department of Neurology, Zealand University Hospital, DK-4000 Roskilde, Denmark (Future study center, in 2019 or 2020)</p> <p>All Danish stroke centers will be invited to participate.</p> |
| <p>Planned study period: 2018-2022.</p> |
| <p>Phase of development: Improve routine care of patients with acute stroke and transitory ischemic attack (TIA).</p> |
| <p>Objectives: To determine whether combined remote ischemic per- and postconditioning can improve long-term recovery in acute stroke patients as an adjunct to standard treatment.</p> |
| <p>Diagnosis: Acute ischemic stroke (AIS) and intracerebral hemorrhage (ICH).</p> |
| <p>Methodology: A multicenter, investigator-driven, prospective, randomized, parallel assignment, patient-assessor blinded, sham-controlled clinical efficacy trial.</p> |
| <p>Randomization:</p> <ul style="list-style-type: none"> • Eligible patients will be randomized prior to the arrival at the hospital (via a secure web site). • The patients and the clinical outcome assessor will be blinded to the treatment allocation. |
| <p>Number and subjects (planned):</p> <ul style="list-style-type: none"> • 1,500 patients |
| <p>Inclusion criteria (prehospital): The initial treatment regime will be applied in the acute prehospital phase in setting of an acute research study</p> <ul style="list-style-type: none"> • Male and female patients (≥ 18 years) • Prehospital putative stroke • Onset of stroke symptoms < 4 hours before remote ischemic conditioning (RIC) • Independent in daily living before symptom onset ($mRS \leq 2$) <p>Final in-hospital inclusion criteria</p> <ul style="list-style-type: none"> • acute ischemic stroke including documented TIA <li style="padding-left: 20px;"><i>or</i> • ICH |

TIA without documented ischemic lesion and non-vascular diagnosis will only have register-based long term follow-up only.

Exclusion criteria (pre-hospital)

Exclusion criteria, to be established during the teleconference between ambulance and on call neurologist

- Intracranial aneurisms, arteriovenous (AV) malformation, cerebral neoplasm, or abscess
- Pregnancy
- Severe peripheral arterial disease in the upper extremities
- AV shunt in the arm selected for RIC
- Concomitant acute life-threatening medical or surgical condition

Criteria for evaluation

Primary endpoints

- Clinical outcome (mRS) at 3 months in acute stroke (AIS and ICH)

Secondary endpoints

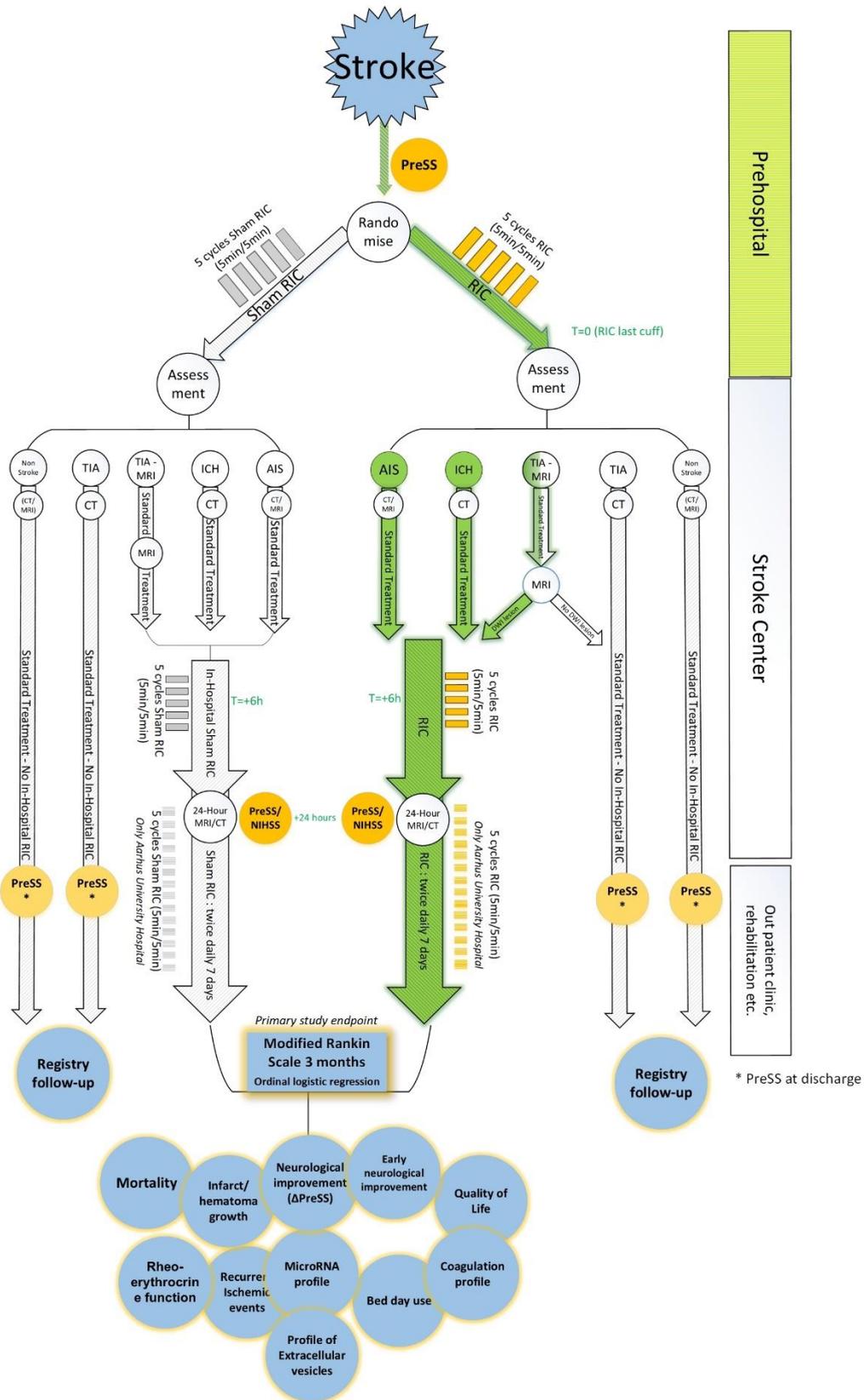
- Difference in prehospital stroke score (PreSS) during 24 hours in all randomized patients
- Clinical outcome (mRS) at 3 months in subgroups: AIS, IV tPA/EVT treated AIS and ICH
- Difference in prehospital stroke score (PreSS) during 24 hours in subgroups: IV tPA/EVT treated AIS and ICH.
- Difference in proportion of patients with complete remission of symptoms within 24 hours (TIA)
- Major Adverse Cardiac and Cerebral Events (MACCE) and recurrent ischemic events based on registry data at 3 and 12 months in ICH, AIS patients, TIA and non-vascular diagnosis
- Three-month and one-year mortality in AIS, ICH, and overall
- Early and very early neurological improvement in AIS and ICH patients
- Bed-day use at 3 and 12 months and quality of life measures at 3 months

Sub-studies at Aarhus University Hospital (secondary endpoints)

- Clinical outcome (mRS) at 3 months in patients with AIS and ICH and extended remote ischemic postconditioning protocol (*substudy at Aarhus University Hospital*)
- Proportion of RIC/Sham-RIC treated AIS patients with large vessel occlusion (LVO) eligible to EVT treatment (*substudy at Aarhus University Hospital*)
- Difference in acute (24-hour) hematoma expansion in patients with ICH
- Difference in hematoma reabsorption rate (7 days) in patients with ICH

- Infarct growth in AIS patients and hematoma growth in ICH patients
- Predictive abilities of Glial Fibrillary Acidic Protein (GFAP) in prehospital obtained blood samples combined with prehospital stroke severity to differentiate hemorrhagic from ischemic stroke and to grade ischemic stroke severity (*substudy at Aarhus University Hospital*)
- Diagnostic abilities of a prehospital microRNA and extracellular vesicles blood samples profile combined with prehospital stroke severity on the differentiation of hemorrhagic from ischemic stroke and to grade ischemic stroke severity (*substudy at Aarhus University Hospital*)
- microRNA and extracellular vesicle profile of RIC-induced neuroprotection at baseline (*substudy at Aarhus University Hospital*)
- Coagulation profile of putative stroke patients in prehospital obtained blood samples (*substudy at Aarhus University Hospital*)
- Modulation of coagulation by RIC (*substudy at Aarhus University Hospital*)
- Characterization of rheoerythrocrine dysfunction (RBC deformability, eryNOS3 and plasma nitrite) in RIC vs Sham-RIC treated stroke patients and its possible association to improved short term (24 hour) or long term (90 day mRS) clinical outcome or imaging biomarkers (DWI infarct growth) (*substudy at Aarhus University Hospital*)
- Prestroke physical activity level (PASE) as a predictor for early and long-term recovery. (*substudy at Aarhus University Hospital*)

Study flow chart



Abbreviations

| | |
|------------|--|
| AE | Adverse event |
| AIS | Acute ischemic stroke |
| AR | Adverse reaction |
| CT | Computed tomography |
| CPSS | Cincinnati Prehospital Stroke Scale |
| DWI | Diffusion-weighted imaging |
| DMC | Data Monitoring Committee and Endpoints Validation Committee |
| ICH | Intracerebral hemorrhage |
| END | Early neurological deterioration |
| EVT | Endovascular treatment |
| LVO | Large Vessel Occlusion |
| miRNA | Micro ribonucleic acid |
| mPTP | Mitochondrial permeability transition pore |
| MRI | Magnetic resonance imaging |
| NPR | Danish National Patient Register |
| PreSS | Prehospital Stroke Score |
| PWI | Perfusion-weighted imaging |
| RIC | Remote ischemic Conditioning |
| RIPerC | Remote Ischemic preconditioning |
| RIPreC | Remote ischemic preconditioning |
| RIPostC | Remote ischemic postconditioning |
| IV tPA | Intravenous thrombolysis/recombinant tissue-type plasminogen activator |
| SAE | Serious adverse event |
| SAR | Serious adverse reaction |
| TIA | Transient ischemic attack |
| TIA (DWI+) | Transient ischemic attack with a DWI-positive lesion on MRI |
| TMG | Trial Management Group |
| TSC | Trial Steering Group |

2. Introduction

Our primary aim is to investigate whether remote ischemic conditioning (RIC) as an adjunctive treatment can improve long-term recovery in acute stroke patients as an adjunct to standard treatment.

Stroke is the second-leading cause of death worldwide and a leading cause of serious, long-term disability. The most common type is AIS which occurs in 85% of cases. Acute cerebral thromboembolism leads to an area of permanent damage (infarct core) in the most severely hypoperfused area and a surrounding area of impaired, yet salvageable tissue known as the “ischemic penumbra”¹. Intravenous alteplase (IV tPA) and endovascular treatment (EVT) are approved acute reperfusion treatments of AIS to be started within the first 4½-6 hours and as soon as possible after symptom onset to prevent the evolution of the infarct core^{2,3,4,5}. The prognosis has improved overall for ischemic stroke and the one-month mortality rate has declined to an approx. 10%⁶. However, reperfusion itself may paradoxically result in tissue damage (reperfusion injury) and may contribute to infarct growth^{7,8,9}. Infarct progression can continue for days following a stroke, and failure of the collateral flow is a critical factor determining infarct growth¹⁰. The pre-eminent therapeutic aim is to restore blood flow, improve collateral perfusion, and prevent reperfusion injury¹¹. In transient ischemic attack (TIA), the ischemia and stroke symptoms are only temporary, but these patients possess a high risk of re-stroke – especially patients with evidence of infarction on neuroimaging¹². TIA patients with a documented ischemic lesion are, per definition, handled as ischemic stroke in this study.

On the other hand, in ICH the culprit is an eruption of blood into the brain parenchyma causing tissue destruction with a massive effect on adjacent brain tissues. Hematoma expansion as well as inflammatory pathways that are activated lead to further tissue damage, edema, and penumbral hypoperfusion¹³. The prognosis after ICH is poor with a one-month mortality of 40%. Acute blood pressure lowering is recommended to prevent further hematoma growth, but the mechanisms behind this effect are not understood¹⁴.

Novel therapeutics and neuroprotective strategies that can be started ultra-early after symptom onset are urgently needed to reduce disability in both AIS and ICH.

Ischemic conditioning is one of the most potent activators of endogenous protection against ischemia-reperfusion injury¹⁵. RIC can be applied as repeated short-lasting ischemia in a distant tissue that results in protection against subsequent long-lasting ischemic injury in the target organ¹⁶. This protection can be applied prior to or during a prolonged ischemic event as remote ischemic preconditioning (RIPreC) and perconditioning (RIPerC), respectively, or immediate after reperfusion as remote ischemic postconditioning (RIPostC)¹⁷. RIC is commonly achieved by inflation of a blood pressure cuff to induce 5-minute cycles of limb ischemia alternating with 5 minutes of reperfusion. RIC activates several protective mechanisms, through humoral and neuronal pathways¹⁸. Circulating microRNA appears to be intimately involved in the RIC stimulus and might act as possible effector molecules^{19,20}.

Preclinical studies show that RIC induces a promising infarct reduction in an experimental stroke model^{21,22}. It has been demonstrated that RIC protects against ischemia-reperfusion injury in the heart, lung, kidney, and the brain²². Results from a recent proof-of-concept study at our institution indicate that RIPerC applied during ambulance transportation as an adjunctive to in-hospital IV tPA increases brain tissue survival after one month²³. Furthermore, RIPerC patients had less severe neurological symptoms at admission and tended to have decreased perfusion deficits²³. Another study using RIPreC found a decreased stroke recurrence and shorter time to recovery in patients with intracranial arterial stenosis^{24, 25}. Furthermore,

emerging preclinical data indicate a significant acceleration of hematoma size reduction increased hematoma reabsorption rate and an improved functional outcome after RIC-treated ICH²⁶.

To-date, no serious adverse events have been documented in RIC. The procedure has been applied in numerous cardiovascular ischemic patients and in patients suffering from cerebral hemorrhage (ICH/SAH)^{23,27,28,29,30}.

RIC is a non-pharmacologic and non-invasive treatment without noticeable discomfort that has first-aid potential worldwide³¹. However, whether combined remote ischemic per- and postconditioning can improve long-term recovery in AIS and ICH has never been investigated in a randomized controlled trial.

3. Hypothesis

RIC as an adjunctive treatment improves long-term functional recovery in AIS and ICH patients.

4. Objectives

Primary objectives

- To determine whether RIC improves the clinical outcome (mRS) at 3 months in AIS and ICH

Secondary objectives

- To determine whether RIC reduces neurological impairment (PreSS) in all randomized patients
- To determine whether RIC improves the clinical outcome (mRS) at 3 months in subgroups of AIS, IV tPA/EVT-treated AIS patients and ICH patients
- To determine whether RIC reduces neurological impairment (PreSS) at 24 hours in subgroups of IV tPA/EVT treated AIS and ICH.
- To determine whether RIC increases the proportion of patients with complete remission of symptoms within 24 hours (TIA; both with and without DWI lesion)
- To determine whether RIC reduces major adverse cardiac and cerebral events (MACCE) and reduces recurrent ischemic events at 3 and 12 months
- To determine whether RIC reduces the 3-month and 1-year mortality in patients with AIS, ICH, and overall
- To determine whether RIC reduces bed-day use at 3 and 12 months and affects quality of life at 3 months
- To determine whether RIC increases the occurrence of early and very early neurological improvement in AIS and ICH patients

Secondary endpoints (only Aarhus University Hospital)

- To determine whether an extended RIC protocol (one week) improves clinical outcome (mRS) at 3 months in patients with AIS and ICH (*substudy at Aarhus University Hospital*)
- To determine whether RIC increased the proportion of AIS patients with large vessel occlusion (LVO) eligible to EVT treatment (*substudy at Aarhus University Hospital*)

- To determine whether RIC reduces infarct growth in AIS patients and hematoma growth in ICH patients (*substudy at Aarhus University Hospital*)
- To determine whether RIC increases the hematoma reabsorption rate (7 days) in patients with ICH (*substudy at Aarhus University Hospital*)
- To determine whether prehospital obtained Glial Fibrillary Acidic Protein (GFAP) in blood samples combined with prehospital stroke severity can differentiate hemorrhagic from ischemic stroke and grade ischemic stroke severity (*substudy at Aarhus University Hospital*)
- To determine whether prehospital obtained microRNA and extracellular vesicles blood samples combined with prehospital stroke severity can differentiate hemorrhagic from ischemic stroke and grade ischemic stroke severity (*substudy at Aarhus University Hospital*)
- To determine whether a circulating microRNA and/or extracellular vesicle profile of RIC-induced neuroprotection can be established (*substudy at Aarhus University Hospital*)
- To determine whether the prehospital determined coagulation profile from putative stroke patients differs between hemorrhagic, ischemic stroke and non-stroke (*substudy at Aarhus University Hospital*)
- To determine whether RIC modulates coagulation in hemorrhagic and ischemic stroke, and explore links between coagulation and infarct growth in AIS, hematoma expansion in ICH, and MACCE and recurrent ischemic events at 3 and 12 months in both AIS and ICH (*substudy at Aarhus University Hospital*)
- To characterize the rheoerythrocyte dysfunction (RBC deformability, eryNOS3 and plasma nitrite) in RIC vs Sham-RIC treated stroke patients and its possible association to improved short term (24 hour) or long term (90 day mRS) clinical outcome or imaging biomarkers (DWI infarct growth) (*substudy at Aarhus University Hospital*)
- To determine whether prestroke physical activity level (PASE) is a predictor for early and long-term recovery (*substudy at Aarhus University Hospital*)

5. Trial design

Trial design

This is a multicenter, prospective, randomized, patient-assessor blinded, sham-controlled trial investigating whether RIC improve long-term recovery in acute stroke.

Number of centers

Patients with putative stroke from participating stroke centers in Denmark will be recruited.

Number of subjects

We estimate that a sample size of 1500 prehospital putative stroke patients will be required to achieve 1000 eligible AIS and ICH patients (primary study endpoint).

Sample size determination

Primary clinical endpoint

The treatment effect of RIC on long-term functional outcome is unknown. We have assumed a small but clinical significant neuroprotective effect corresponding to a 6-7% increased odds for a beneficial shift on the modified Rankin Scale. The sample size calculation was based on a simulation-based approach to the analysis of statistical power when ordinal logistic regression analysis is performed (significance level of 5%)

The statistical power was simulated at different hypothetical sample sizes (on the target population) (ranging from 200 to 1900) with 2000 simulation-runs performed at each step. Unpublished data on IV-tPA and/or EVT treated AIS patients and ICH patients from our institution were used:

3 months modified Rankin Scale distribution (proportions) in 2017 for patients with ICH or IV tPA/EVT treated AIS at Aarhus University Hospital.

| Modified Rankin Scale score | mRS 0 | mRS 1 | mRS 2 | mRS 3 | mRS 4 | mRS 5 | mRS 6 |
|-----------------------------|-------|-------|-------|-------|-------|-------|-------|
| Proportion | 0.139 | 0.273 | 0.141 | 0.110 | 0.145 | 0.07 | 0.126 |

Based on our previous trial experience with prehospital remote ischemic conditioning we estimate that a sample of 1000 subjects with target diagnosis (AIS and ICH) will be feasible to include during the study period.

Including 1000 patients with target diagnosis provide sufficient power at a significance level (alpha-level) of 5% to detect RIC treatment effects of the estimated 6-7 % (see table below)

| Treatment effect (assumed neuroprotective) | 5% | 6% | 7% |
|--|------|-------------|-------------|
| Sample (target diagnosis), <i>n</i> | 1000 | 1000 | 1000 |
| Power | 66% | 80% | 90% |
| Alpha level (significance level) | 5% | 5% | 5% |

The estimated prehospital, randomized, sample:

| Sample size, prehospital | Proportion of randomized | <i>n</i> = |
|---|--------------------------|-------------|
| Target diagnosis (AIS and ICH), <i>n</i> | 67% | 1000 |
| Non-vascular diagnosis | 27% | 403 |
| TIA without DWI lesion | 4% | 60 |
| Lost to follow-up | 2% | 30 |
| Total | | 1492 |
| Plan to include | | 1500 |

We therefore plan to include 1500 patients with a prehospital putative stroke in order to get 1000 patients with the target diagnosis of acute ischemic stroke and intracerebral hemorrhage. There is no planned replacement of patients lost to follow-up.

Randomization

Randomization procedure

The patient will be randomized to standard treatment with RIC or sham-RIC by the on call neurologist/vascular neurologist at the receiving stroke center. The ambulance will contact the on call neurologist by telephone and describe the patient (standard operating procedure (SOP) in Denmark). The randomization is based on a secure web site providing computer-generated blocked randomization lists stratified by the center. The online randomization is stratified by age, strokecenter and the Prehospital Stroke Score (PreSS). The PreSS score consists of the Cincinnati Prehospital Stroke Scale (CPSS) with an additional opportunity to report other neurological symptoms (e.g. ataxia, sensory disturbances and visual field loss), and PASS (Prehospital Acute Stroke Severity Scale)^{32,33}. Prehospital personnel participating in RESIST are trained in identifying stroke symptoms included in PreSS. The on call neurologist will make an assessment based on all available information whether the patient is eligible to participate in RESIST. Randomization is performed in the prehospital setting. Each on call neurologist participating in the study will receive unique access and will have no influence on the randomization process. All neurologists on call will be educated and trained in performing evaluation of potential eligible study candidates and to perform the online randomization during the telephone call with the ambulance personnel. There is always one neurologist on call in participating centers (hospital SOP).

Treatment allocation

1:1 Allocation

6. Selection and withdrawal of subjects

Selection of patients

All patients with a putative stroke who meet the study criteria will be included.

- Depending on the prehospital randomization (RIC versus sham-RIC), patients with AIS will continue RIC or sham-RIC treatment for an extended period. This group includes TIA with a documented DWI lesion on magnetic resonance imaging (MRI).
- Patients with ICH will continue in-hospital RIC or sham-RIC (according to randomization).

Informed consent for prehospital enrollment

Background:

Acute temporary cognitive impairment after stroke is very common, even in patients with minor stroke or remission of symptoms (TIA) (54%)³⁴. Widespread cognitive deficits have been identified in an acute stroke patient population³⁵. This finding is in line with the clinical experience in acute stroke care. Whether the putative stroke patients have major cognitive impairment in the prehospital setting can only be tested under calm and quiet in-hospital circumstances. Delivering acute neuroprotective care, it is of utmost importance that treatment is started as soon as possible, i.e. without delay. RIC is without any known serious adverse events.

According to the Danish research ethics committees, a patient who is a candidate for inclusion in an acute research study is considered incompetent if that person is not able to care for his or her own affairs due to physical or mental impairment. We believe that the vast majority of stroke patients fulfil these criteria, and it is not possible to select the few who are, indeed, competent in the acute phase without formal cognitive testing and thus missing the purpose of acute neuroprotection intervention. The intervention is associated only with mild to moderate discomfort and minimal risk for adverse events.

Acute Prehospital phase:

The initial RIC/sham-RIC treatment regime will be applied in the acute prehospital phase in setting of an acute research study approved by the regional research ethics committees.

Whether further treatment, according to randomization, will be offered depends on the in-hospital examination.

In-hospital inclusion:

After the initial RIC/sham-RIC treatment, in-hospital diagnostic evaluation, and acute treatment, **the competent patient** will be presented with a consent form with information about the study. Before this, the on-duty neurologist/research physician will have examined the patient both physically and cognitively and will have assessed whether the patient is competent or not. The competent patient will receive oral information by the on-duty neurologist/research physician based on the written patient information. The written patient information will be handed out (*deltager information*). We will ask for the patient's acceptance to be included in the study. The patient can withdraw consent at any time. In addition, the acute nature of the disease necessitates circumvention of the usual requirements concerning a 24-hour time period for consideration and discussion with a lay representative. This has been done in recent comparable acute studies. This approach is acceptable as the intervention is without any known risk and associated with a potential benefit for the patient. In-hospital inclusion and assessment of cognitive impairment will be undertaken during the admission at the stroke center. Every effort will be made to inform the patients and relatives in quiet and undisturbed settings, this will be at the hospital bedroom or the designated room for conversations. We will prioritize the presence of nearest relatives at time of study information and inclusion.

For **the incompetent patients**, informed consent will be obtained from next of kin and an independent physician. The consenters will be provided information (based on the summary of the protocol for the independent physicians and study participant information for the relatives) on the trial to be able to make an informed decision about the patient's participation in this trial. The independent physician will be the on-duty physician at the local department of neurosurgery, neuroanesthesiology, intensive care, neurophysiology, (neuro)radiology or the on duty physician from another specialty with stroke patient experience. On-duty physicians at these departments will receive detailed study information.

A patient who during the acute phase was legal incompetent and included as such, but during the follow-up period is assessed competent will receive oral and written information and we will ask for his/her's informed consent.

Patients who fulfill both prehospital and in-hospital inclusion criteria will, according to randomization, receive RIC/sham-RIC treatment again after +6 hours (**all centres**).

Participation in the extended remote ischemic conditioning(RIC) or sham-RIC (*for a total of 7 days*) study will take place **only at Aarhus University Hospital**. The device will follow the patient when he/she is discharged from the stroke center.

TIA patients (without DWI lesion) and non-stroke diagnosis patients will be asked for consent to do registry follow-up until one year after discharge. For these patients end-of-study visit is registered at stroke center discharge.

Inclusion criteria (prehospital)

- Male and female patients (≥ 18 years)
- Prehospital putative stroke
- Onset of stroke symptoms < 4 hours before RIC
- Independent in daily living before symptom onset ($mRS \leq 2$)

Exclusion criteria (prehospital)

- Intracranial aneurisms, AV malformation, cerebral neoplasm or abscess
- Pregnancy*
- Severe peripheral arterial disease in the upper extremities
- Concomitant acute life-threatening medical or surgical condition
- AV shunt in the arm selected for RIC

**Women of child-bearing age should be asked about their use of safe birth control methods (contraceptive pill, intrauterine devices both hormonal and non-hormonal, hormonal implants, hormonal depot injection and transdermal hormonal patch). If pregnancy cannot be ruled out in the prehospital phase the patient can't be included. Women with a safe birth control method should be encouraged to use this method during the entire period of active RIC treatment.*

Final in-hospital inclusion criteria

- AIS (including DWI-positive TIA patients)
- or*
- ICH

TIA and non-stroke diagnose will only have long-term register based follow-up.

Method of blinding

Outcome assessment is blinded to the treatment arm and obtained by a clinical or telephone assessment of the level of dependency and need for help in daily activities (**modified Rankin Scale**) (**Primary study endpoint**).

No information regarding randomization status will be recorded in the patient record.

Patients and the assessors of endpoints are blinded.

Discontinuation of study participation

A patient can withdraw from the study at any time. Patients can be withdrawn from the study at the principal investigator's discretion. In case the patient cannot be reached by telephone, every effort will be made to contact the patient or to document the outcome regarding new vascular events via

registries/electronic health records. If the patient withdraws from the study, the date and the reason for the patient's withdrawal will be recorded. The patient is encouraged to provide information about his or her reason(s) for withdrawal and any experienced adverse effects (AE) during the study.

7. Study procedure and assessment

Prehospital stroke identification

Prehospital putative stroke patients are identified in collaboration with the ambulance personnel and the on-duty neurologist/vascular neurologist at the nearest stroke center (by telephone), which is standard operating procedure in Denmark. All Danish ambulance personnel are trained in identifying putative stroke in the prehospital setting. In the present study protocol, all patients have a prehospital structured assessment of neurological symptoms/findings (based on items contained in PreSS) before randomization – which will further increase stroke symptom awareness.

Baseline data

Baseline data and process indicators are collected from the Danish Stroke Registry (DSR). Additional information about medical history and medication and clinical and physiological data are collected at baseline. Data are recorded in an electronic case report form (CRF) at discharge from the stroke unit. For a complete list of registered data, see *Supplement B*

Acute treatment:

Patients are treated according to the national clinical guidelines on stroke treatment, intravenous thrombolysis and endovascular therapy. All patients are treated with state-of-the-art multidisciplinary care in the setting of a stroke unit monitored by the DSR.

Blood samples

Patients with putative stroke admitted to the Aarhus University Hospital will have blood samples withdrawn, in the ambulance, from peripheral venous catheter (placed as standard operating procedure, SOP). A total of 19mL (9mL + 10mL) will be drawn in the prehospital phase.

Blood samples will also be drawn upon admission to the Aarhus University Hospital Stroke Center as a part of SOP. Additional 19mL (9mL + 10mL) of blood will be drawn at this time.

Patients with AIS and ICH diagnosis will have blood-samples drawn, at Aarhus University Hospital Stroke Center, again 24 (16-32) hours after randomization. This is not standard operating procedure. 19mL (9mL + 10mL) will be drawn at this time.

Only patients received at the Stroke Center at Aarhus University Hospital will have blood samples drawn. (Prehospital and in-hospital blood samples)

Not all ambulances will be equipped with for blood sample withdrawal, thus an estimated 500 patients will have blood samples drawn in the prehospital phase and in hospital at Aarhus University Hospital.

Blood samples will be stored until completion of the trial in a research biobank.

Patients not admitted at Aarhus University Hospital will have no additional blood samples drawn (neither prehospital or in-hospital)

MicroRNA, extracellular vesicles, Glial Fibrillary Acidic Protein (GFAP), RBC deformability, NOS3, plasma nitrite and coagulation analysis will be performed on the obtained blood. A research bio bank will be established, and all blood samples obtained during the study are stored here. During the entire study and analysis period, the material will be stored at Aarhus University Hospital. The last patient will be included in December 2022. Analysis of blood samples, according to secondary endpoints, will be performed during the study and until 2 years after inclusion of the last patient (no later than 31. December 2024). Hereafter the remaining material will be transferred to a biobank for future unspecified research purposes. We will submit an application to the Danish Data Protection agency for this additional 20-year storage period of the blood samples (until 31 December 2044), after that, the samples will be destroyed. All analysis will be performed in Denmark.

Investigational devices and Remote ischemic conditioning protocols

Automatic remote ischemic conditioning devices

Both the RIC and sham-RIC device are developed in collaboration with Aarhus University, Faculty of Biomedical Engineering, 8200-DK, Aarhus N, Denmark; Seagull Aps, 4200-DK, Slagelse, Denmark and the Department of Neurology, Aarhus University Hospital, 8000-DK, Aarhus C, Denmark.

The devices are manufactured by Shenzhen Raycome Health Technology Co., Ltd, 3F, 51 Building, No.5 Qiong Yu Road, Hi-Tech Industrial Park, Nanshan District, Shenzhen, China 518057.

*The **Investigator's Brochure (IB)** contains a detailed description of the investigational devices including risk analysis assessment.*

A brief user's manual is found in each device bag. No preexisting experience is necessary in order to operate the device. Written and oral information combined (Healthcare professional at participating hospitals) with RESIST trial e-learning material (prehospital personnel) will be offered to familiarize the clinical personnel in using the investigational devices. Patients and relatives will be instructed using written and oral information.

Remote Ischemic Conditioning device (RIC device)

The device is programmed to five cycles (50 minutes), each consisting of five minutes of cuff inflation followed by five minutes with a deflated cuff. The cuff pressure will be 200mmHg; but if initial systolic blood pressure is above 175 mmHg, the cuff is automatically inflated to 35 mmHg above the systolic blood pressure. This is done to account for a maximum cuff air leakage of 6mmHg per minute and maximum deviation in bloodpressure measurement of 10mmHg. The maximum cuff pressure is 285 mmHg.

Sham – remote ischemic conditioning device (Sham-RIC device)

The device is programmed to five cycles (50 minutes), each consisting of five minutes of cuff inflation followed by five minutes with a deflated cuff. The cuff pressure during inflation will be 20mmHg.

Timestamps, blood pressure before RIC/sham-RIC and total RIC/sham-RIC cycles are recorded and stored on the device (accessible by *universal serial bus*, USB). The RIC and sham-RIC is pre-programmed before

being delivered to collaborating centers/ambulances. No data on conditioning cuff pressure will be displayed on device screen.

In cases of short transport time, the RIC/sham-RIC protocol continues during the initial assessment at the stroke center. RIC/sham-RIC stimulation is discontinued just prior to the MRI and continues afterwards if the protocol was incomplete (< 5 cycles). In centers using CT, the RIC/sham-RIC protocol is continued during the scan until a total of 5 cycles are reached. The device automatically stops treatment when the 5 cycles are completed. Except for RIC/sham-RIC, the prehospital observation and treatment are according to standard procedures in the ambulance.

Stroke RIC/Sham-RIC will be used, only if approved by the Danish Medicines Agency

The blood pressure and heartrate measurement, by RIC and Sham-RIC, will only be used for research purposes.

Initial remote ischemic conditioning

(all patients)

RIC/sham-RIC cuff will be placed on the non-paretic upper arm. The cuff is placed on the upper extremity on the same side of the suspected side of cerebral stroke, e.g. in right hemiparesis the cuff on the left arm, and in monosymptomatic aphasia the cuff is placed on the left arm.

Remote ischemic conditioning at +6 hours

(All patients with AIS, ICH – all centers)

At 6 hours after the first RIC/sham-RIC protocol (6 hours from the last cuff-off, respectively), another series of RIC/sham-RIC is performed; five cycles each consisting of 5 minutes of ischemia are followed by 5 minutes of reperfusion.

Remote ischemic postconditioning (twice daily for 7 days)

(AIS and ICH – only at Aarhus university Hospital)

The post-conditioning stimulus will be applied twice daily in the first week. This occurs at pre-specified times: 08.00 PM and AM. However, RIC/sham-RIC can be applied no sooner than 10 hours and not later than 21 hours after the initial preconditioning stimulus (last cuff).

Aarhus University Hospital: RIC/sham-RIC treatment will continue for a total of 7 days. If patients are discharged to their home or to another department, they will continue treatment according to written instructions, including application of the device for 7 days. After RIC/sham-RIC on day 7, the device is then returned to the Stroke Center.

| RIC/sham-RIC protocol | Accepted time frame |
|--|---|
| Prehospital RIC | < 4 hours from symptom onset |
| RIC at +6 h | 4 to 8h from last cuff of the initial RIC |
| RIC twice daily for 7 days – 08.00 PM and AM* | 06.00 to 10.00 PM and 06.00 to 10.00 AM |

*RIC/sham-RIC can be applied no sooner than 10 hours and no later than 21 hours after the initial preconditioning stimulus.

Device Accountability log

All devices are registered in a *device accountability log*. When the prehospital personnel arrives at the stroke center, with a patient included in RESIST trial, a new device (of the same color) will be handed out. Device ID, ambulance ID, contact details to the ambulance and date of device hand-out will be registered. It will be recorded on a paper-log in the stroke department. These data are transferred to an electronic log keeping track of device location and time to service/battery charge (1 year). *Further details see Investigators Brochure.*

Study procedures table

| | Acute prehospital and in-hospital phase | | | | | + 6 hour | + 24 hour | | | + 7days | +3 months | 12 months | |
|-------------------------------|--|------------------------------------|--------------|-------------------------------------|-----------------|-----------------------------------|------------------------|-------------------------|-----------------------|---|-------------------------|---------------------------|---|
| | Prehosp. stroke score (PreSS) ¹ | Prehosp Blood-samples ¹ | RIC/Sham-RIC | In-hospital assessment ² | CT/MRI baseline | +6 hour RIC/sham-RIC ⁴ | PreSS 24h ² | CT/MRI 24h ³ | 24-hour Blood-samples | Extended (7 days) RIC/sham-RIC ⁵ | CT- 7 days ³ | mRS 3-months ⁷ | Clinical events during follow-up ⁶ |
| TIA (DWI-) - all | X | (X)* | X | X | X | | X | | | | | | X |
| Non-stroke - all | X | (X)* | X | X | X | | X | | | | | | X |
| AIS incl. TIA (DWI+) – Other‡ | X | | X | X | X | X | X | X | | | | X | X |
| ICH - Other‡ | X | | X | X | X (CT) | X | X | | | | | X | X |
| AIS incl. TIA (DWI+) – AUH* | X | (X) | X | X | X(MRI) | X | X | X (MRI) | (X) | X | | X | X |
| ICH – AUH* | X | (X) | X | X | X | X | X | X (CT) | (X) | X | X (CT) | X | X |

*AUH = Aarhus University Hospital , ‡ Other= Other participating stroke centers

¹ Prehospital stroke score (PreSS) and bloodsamples. *Prehospital blood samples (19mL ~~2x5.5mL~~): only patients transported to Aarhus University Hospital)*

² Acute assessment at the Stroke Center. NIHSS, blood samples (19mL blood) (~~2x5.5mL blood~~) and ECG (standard). Assessment of neurological symptoms at 24-hour: NIHSS. Patients with non stroke diagnosis and TIA without a DWI-MRI lesion, who are discharged before 24 hours will be scored using PreSS at discharge (~~performed by the on-call neurologist~~). Patients with vascular diagnosis, who had blood samples taken in the ambulance and upon stroke center arrival will have additional samples withdrawn at 24 hour (additional 19mL blood). *In-hospital RESIST blood samples only at Aarhus University Hospital*

³ Baseline CT is preferable for ICH patients (hospital SOP). ICH patients, at Aarhus University Hospital, will receive a 24 hour control CT (hospital SOP). If possible, a 24-hour MRI is performed in all included AIS patients with an MRI baseline scan. Furthermore, a 24-hour control CT will be provided for IV tPA-treated AIS patients, unless MRI can be performed and was the primary investigation. One week control CT will be performed, if possible, on ICH patients included at Aarhus University Hospital. End of study visit for non-vascular diagnosis and TIA (without DWI lesion) at stroke center discharge.

⁴ RIC/sham-RIC at +6 hours from last RIC cuff

⁵ RIC/sham-RIC twice daily for 7 days (*Only at Aarhus University Hospital*)

⁶ Mortality, MACCE, and recurrent ischemic events are recorded using the Danish National Patient Register (LPR) and DSR at 6 and 15 months after the inclusion of the last patient.

⁷ Assessments at 3 months by telephone or face-to-face (mRS and WHO-5). End-of-study (telephone or face-to-face) for AIS and ICH patients. WHO-5 will only be assessed by one assessor.

8. Endpoints

An independent end-point committee will adjudicate clinical events.

Primary endpoints

Criteria for evaluation

Clinical outcome (mRS) at 3 months in acute stroke

Primary study endpoint: Clinical outcome (modified Rankin Scale) at 3 months in acute stroke patients (generalized ordinal logistic regression)

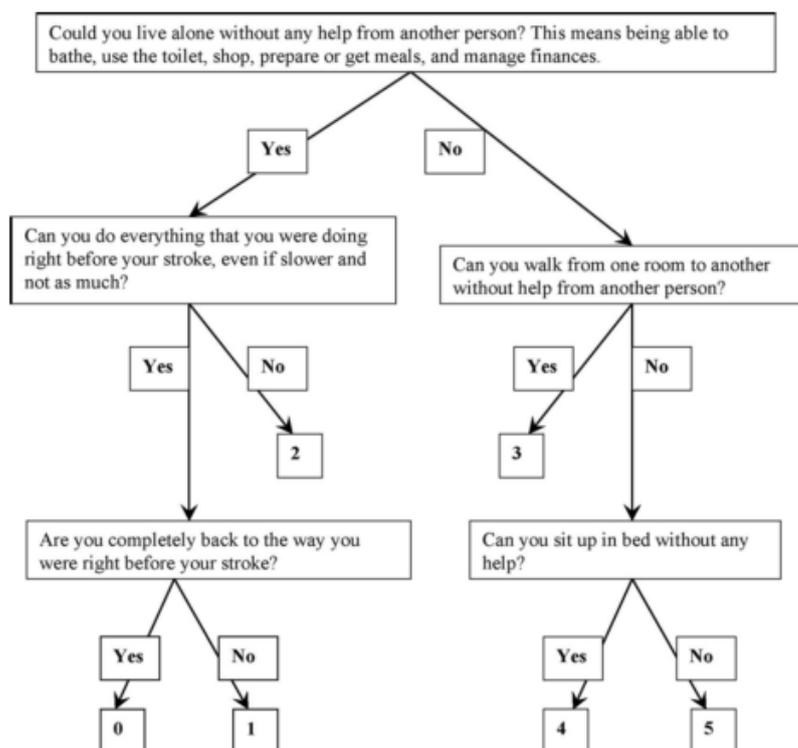
The level of dependency and need for help in daily activities will be determined by either face-to-face assessment or based on a structured telephone interviews performed by assessors who are blinded to the intervention at 3 months after the index stroke.

The assessment will be performed by two independent blinded assessors, which can be either face-to-face or telephone based assessment (not same day). The electronic CRF database will alert if disagreement occurs, and the patient will be contacted by a third assessor (face-to-face or telephone) who is blinded to the intervention who will assess the level of dependency.

- If disagreement occurs between **two telephone assessments** – a third, and final, telephone or face-to-face assessment will be made.
- If disagreement occurs between one face-to-face assessment and one telephone assessment – the face-to-face will be considered the final assessment
- If disagreement occurs between **two face-to-face assessments** – a third, and final, telephone or face-to-face assessment will be made.

Every possible effort will be made to assess the outcome in patients who are unable to participate in the interview (this could be due to post stroke neurological impairment (for example aphasia and/or dysarthria), or that the patient cannot be reached by telephone). The outcome will then be assessed by contact to a named relative or the general practitioner. Several attempts to contact the patient by telephone will be made before contacting the named relative or general practitioner. Death in the follow-up period will be obtained from Danish Civil Registration System (CPR).

The structured telephone interview will be based upon a validated Danish translation of the “slightly revised simplified, modified Rankin Scale questionnaire”³⁶. The translation and its validation will be performed according to the AAOS guideline^{36, 37}



Bruno et al. 2011. Stroke.

Furthermore, modified Rankin Scale assessors are required to have received specific mRS training, which could be an online mRS certification <https://secure.trainingcampus.net/uas/modules/trees/windex.aspx?rx=rankin-english.trainingcampus.net>

For a **detailed statistical analysis plan**, see **statistics**

| Outcome | Accepted time frame (from symptom onset) |
|--|--|
| 3-month follow-up – 1 st and 2 nd assessment | 3 months +/- 2 weeks |
| 3 month follow-up – 3 rd assessment | 3 months and 2 weeks +/- 2 weeks |

Secondary endpoints

Difference neurological impairment during the first 24 hours in all randomized patients

Neurological deficits are documented using PreSS (prehospital and in-hospital). The PreSS score is obtained by the prehospital personnel and registered by the on-call neurologist before randomization. Prehospital Stroke Score is assessed at 24-hour by a certified, trained and blinded general neurology nurse/neurology research nurse/physician/neurologist who is blinded to the treatment intervention and who is not a part of the daily stroke ward routine. Only information regarding time and place of assessment, patient name, age

and social security number will be available. The PreSS score of patients with TIA (without DWI lesion) and non stroke diagnoses, are performed by the on-call physician/neurologist or a research nurse/general neurology nurse, and will be documented as *PreSS at discharge*, if discharge occurs before 24 hours. The outcome assessor will be alerted in advance of the assessment. The PreSS is recorded in the electronic CRF. Clinical assessors are required to complete an online PreSS certification or face-to-face training.

https://ekurser.rm.dk/test_projekter/anhesr/red-hjernen/scorm/Scorm2004%204th%20Edition/index.html

For a **detailed statistical analysis plan**, see **statistics**

| Indicator | Accepted timeframe (from symptom onset) |
|------------------------------|---|
| PreSS | Close to symptom onset |
| PreSS _{24h} (+ 24h) | + 16 to 32h |

Difference in proportion of patients with complete remission of symptoms within 24 hours (TIA; both with and without DWI)

Diagnosis of TIA is documented in the electronic case report form

Endpoint assessment

Difference in proportion of patients with complete remission of symptoms within 24 hours (TIA; both with and without DWI)

Major adverse cardiac and cerebral events (MACCE) and recurrent ischemic events at 3 and 12 months in AIS and all patients

MACCE is defined as:

Cardiovascular events (cardiovascular death, myocardial infarction, acute ischemic or hemorrhagic stroke)

Cardiovascular death: Death from known cardiovascular cause or sudden death from unknown cause (no identified cause of death in medical history and/or autopsy)

Acute myocardial infarction: Admission with a discharge diagnosis of ST-elevation myocardial infarction (STEMI) and non-ST elevation myocardial infarction (NSTEMI) and unstable angina pectoris (UAP)

Stroke: Admission with a discharge diagnosis of acute ischemic or hemorrhagic stroke.

Evaluation is performed using the Danish National Patient Register (LPR) and the DSR at two time points (6 and 15 months after the inclusion of the last patient).

Diagnosis of AIS/TIA, ICH and MI (STEMI, NSTEMI, and UAP) are made according to national clinical practice guidelines.

(<http://neuro.dk/wordpress/nbv/om-iskaemisk-apopleksi/> and <http://nbv.cardio.dk/aks>)

Recurrent ischemic vascular events at 3 and 12 months in AIS patients

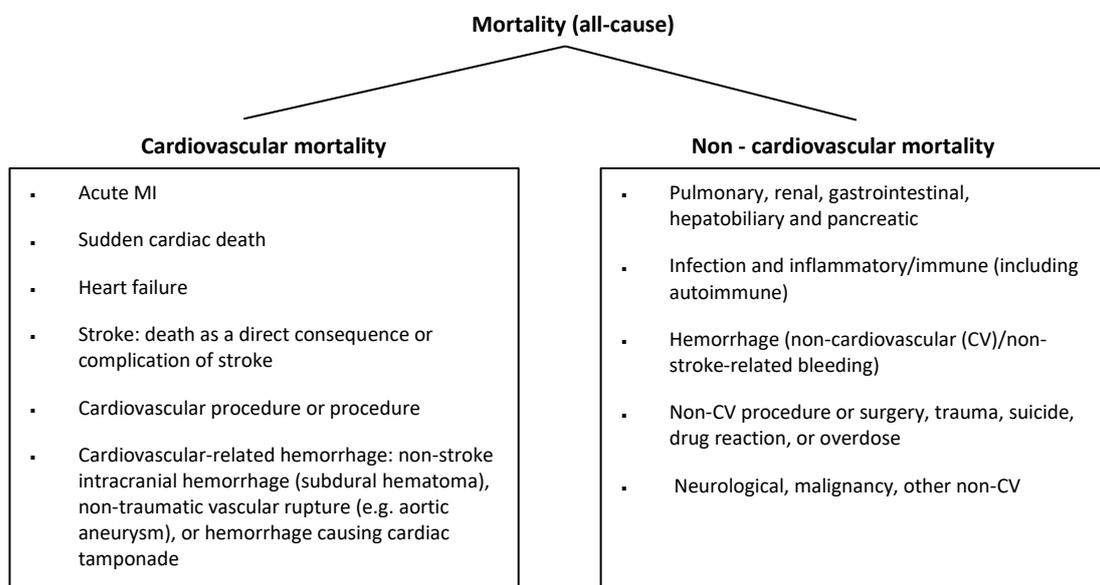
Recurrent ischemic vascular events defined as:

- AIS
- TIA
- MI, STEMI, and NSTEMI

Three-month and one-year mortality

Information about mortality is collected using the CPR and LPR, at two time points (6 and 15 months after the inclusion of the last patient). All-cause mortality is assessed and subdivided into cardiovascular mortality versus non-cardiovascular mortality⁴⁰.

Analysis are performed on all randomized patients and according to subgroups.



According to “2014ACC/AHA Key Data Elements and Definitions for Cardiovascular Endpoint Events in Clinical Trial”. Circulation 2015;132:302-361.

EVT-eligibility (MRI assessed) in RIC treated AIS patients with large vessel occlusion(LVO)

EVT eligibility is assessed upon arrival at the Aarhus University Hospital in AIS patients with:

- Severe Stroke (NIHSS ≥ 10)
- Groin puncture feasible within 6 hours from stroke onset
- MRI-TOF (time-of-flight) documented internal carotid artery (ICA), Intracranial ICA (ICA-T) and first and second stem of the middle cerebral artery (M1 and M2, respectively)
- No contraindications to MRI (pacemaker, vomiting, respiratory insufficiency, obesity)
- MRI-DWI lesion volume $\leq 70\text{mL}$

Endpoint assessment

Proportion of RIC treated AIS patients with LVO eligible to EVT treatment compared to standard treatment, adjusted for prehospital stroke severity (PreSS) and symptom duration

Difference in MRI-DWI lesion volume in RIC/sham-RIC treated LVO AIS patients eligible to EVT treatment

Quality of life measures at 3 months in AIS and ICH patients

Quality of life and bed-day use measures in AIS and ICH patients

Quality-of-life measurements are assessed by telephone interview at 3 months after inclusion. The WHO-5 is used. Baseline WHO-5 is only obtained at Aarhus University Hospital.

[Link to: Danish National Board of Health - WHO-5.](#)

Information about bed-day use via the LPR and the DSR.

Early neurological improvement in AIS and ICH patients

Neurological deficits are documented with the PreSS and the National Institute of Health Stroke Scale (NIHSS), prehospital and in-hospital, respectively.

Very early neurological improvement is defined as:

Reduction in prehospital stroke score (PreSS) ≥ 1 points or resolution of symptoms at admission: Items on PreSS related to NIHSS baseline $\Delta\text{PreSS} = \text{PreSS}_{\text{prehospital}} - \text{same items on NIHSS}_{\text{baseline}}$

Early neurological improvement is defined as:

Reduction in NIHSS ≥ 4 (baseline versus 24-Hour NIHSS): $\Delta\text{NIHSS} = \text{NIHSS}_{\text{baseline}} - \text{NIHSS}_{24}$

- **Reduction in prehospital stroke score (PreSS) ≥ 1 points or resolution of symptoms after 24 hours in subgroups: IV tPA/EVT treated AIS and ICH.**

Clinical assessors are required to complete an online PreSS certification

PreSS certification: https://ekurser.rm.dk/test_projekter/anhesr/red-hjernen/scorm/Scorm2004%204th%20Edition/index.html

| Indicator | Accepted timeframe (from symptom onset) |
|--|---|
| PreSS | Close to symptom onset |
| NIHSS baseline | 0 to 6 hours from admission |
| NIHSS (+ 24h) – standard operating procedure | + 16 to 32h |
| PreSS (+ 24h) | + 16 to 32h |

Secondary endpoints (Only Aarhus University Hospital)

Difference in acute hematoma expansion in patients with ICH

(Substudy only at Aarhus University Hospital)

- Hematoma expansion is defined as the difference in volume between the baseline and the 24-hour CT hematoma volume. Significant hematoma growth is defined as an absolute growth exceeding 6 mL or a relative growth of more than 33% from the initial CT³⁹.

In all ICH patients, the baseline imaging modality is CT. In cases of initial MRI, a CT is performed afterwards (no later than < 3 hours from last RIC cuff)

| Imaging protocol | Accepted time frame |
|----------------------------|---|
| CT baseline – ICH | < 3 hours from last RIC/sham-RIC cuff |
| MRI baseline – ICH | Perform CT as soon as possible and < 3 hours after the last RIC/sham-RIC cuff |
| Control CT (24-hour) – ICH | 18-30 hours from baseline CT |

Information about imaging protocols, see *Supplement A – Neuroimaging protocols*

Difference in 7 days hematoma reabsorption rate in patients with ICH

(Substudy only at Aarhus University Hospital)

- Hematoma reabsorption rate is defined as the difference in hematoma volume between the baseline and the 7-day CT hematoma volume.

In all ICH patients, the baseline imaging modality is CT. In cases of initial MRI, a CT is performed afterwards (no later than < 3 hours from last RIC cuff)

| Imaging protocol | Accepted time frame |
|--------------------------|---|
| CT baseline – ICH | < 3 hours from last RIC/sham-RIC cuff |
| MRI baseline – ICH | Perform CT as soon as possible and < 3 hours after the last RIC/sham-RIC cuff |
| Control CT (7-day) – ICH | 5-9 days from baseline CT |

Information about imaging protocols, see *Supplement A – Neuroimaging protocols*

Infarct growth in AIS patients

(Substudy only at Aarhus University Hospital)

Acute infarct growth is defined as the difference in infarct volume between baseline and 24 hours on DWI-MRI. All patients with IV-tPA and/or EVT treated ischemic stroke and a baseline MRI will have an additional 24-hour MRI performed.

| Imaging protocol | Accepted time frame |
|--|---------------------------------------|
| MRI baseline – AIS IV tPA eligible | On admission |
| MRI baseline – AIS not IV tPA eligible | < 6 hours from last RIC/sham-RIC cuff |
| MRI (24-hour) – AIS IV-tPA/EVT treated | 18-30 hours from baseline MRI |

Information about imaging protocols, see *Supplement A*

Biochemical profile of stroke subtypes and of RIC-induced neuroprotection

(Substudy only at Aarhus University Hospital)

Prehospital biochemical substudies (Aarhus University Hospital)

- Diagnostic abilities of a prehospital microRNA and extracellular vesicles blood samples profile combined with prehospital stroke severity to differentiate hemorrhagic from ischemic stroke and to grade ischemic stroke severity
- Predictive abilities of Glial Fibrillary Acidic Protein (GFAP) in prehospital obtained blood samples combined with prehospital stroke severity to differentiate hemorrhagic from ischemic stroke and to grade ischemic stroke severity

- Coagulation profile in prehospital obtained blood samples to differentiate between hemorrhagic stroke, ischemic stroke, and non-stroke
- Red blood cell rheo-erythrocyte dysfunction in acute stroke and its possible association to improved early (24 hour) or long term (90 day mRS) clinical outcome or imaging biomarkers (DWI infarct growth) (*substudy at Aarhus University Hospital*)

In-hospital biochemical substudies

- Characterization of a possible microRNA and extracellular vesicle profile in stroke subtypes
- MicroRNA and extracellular vesicle characterization of a possible RIC treatment profile
- Modulation of coagulation by RIC in hemorrhagic and ischemic stroke
- Characterization of rheoerythrocyte dysfunction (RBC deformability, eryNOS3 and plasma nitrite) in RIC vs Sham-RIC treated in acute stroke patients

Prehospital and in-hospital handling of blood samples:

Blood samples will be collected from patients with a prehospital putative stroke. Thus, blood samples will be drawn in the ambulance and upon arrival at the stroke center (only Aarhus University Hospital). In the ambulance, the blood sample will be obtained through a peripheral venous catheter (placed as standard operating procedure) into a 9mL and 10mL Sarstedt Monovette® tubes and transported to hospital at ambient temperature (Tubes of 9 and 10mL will be drawn). The local biochemical department or study personnel will centrifuge the blood and divide the plasma into cryotubes that will be kept at 80 °C for long-term storage. Cryotubes not used to measure GFAP within 5 to 7 days will be stored in a research biobank.

Blood samples will be drawn upon admission to the Aarhus University Hospital Stroke Center as a part of local stroke center SOP. Additional 19mL (9+10mL) of blood will be drawn at this time.

Patients with AIS and ICH diagnosis will have blood-samples drawn, at Aarhus University Hospital Stroke Center, again 24 (16-32) hours after randomization (19mL (9+10mL)) will be drawn at this time).

All blood samples obtained during the study will be stored in a research biobank

Blood-samples, in the research biobank, will be stored until completion of the trial (no later than 31. December 2024). Hereafter the remaining material will be transferred to a biobank for future unspecified research purposes (see section “Blood Samples” under “study procedure and assessment”)

Planned biochemical analysis

Glial Acidic Fibrillary Protein (GFAP):

All plasma samples for GFAP measures will be analyzed with ELISA assay kits from the same batch at Clinical Biochemical Department, Regional Hospital Herning. Transport and shipment of samples will be done on dry ice to assure stability of the specimens. The samples will be marked with the unique research identification number. Cryotubes not used to measure GFAP will be stored as part of a biobank.

MicroRNA analysis:

MicroRNAs will be identified with Illumina next-generation sequencing using the TruSeq Small RNA Sample Preparation kit (Illumina) which allows for the addition of unique barcode sequences to each sample. Such barcoding allows pooling and simultaneous sequencing of multiple samples in a single-sequencing run on

the Illumina NextSeq500 sequencer thereby significantly reducing the cost of sequencing. Pooling of multiple samples will generate adequate data amounts for detailed miRNA profiling, yet at limited costs. Data generated from the NextSeq500 sequencer will be filtered based on sequence quality as part of our established bioinformatics pipeline. This will also include matching the filtered data to annotated RNA databases such as miRNA sequences from miRBase (mirbase.org).

The output from our bioinformatic pipeline will be quantitative miRNA expression levels for each sample, which will form the basis for an miRNA differential analysis where miRNAs with statistically significant expression changes will be found.

The microRNA (and extracellular vesicles) analysis is not a mapping of the genome. The microRNA profile is a snapshot of microRNA activity at the specific time of the blood sampling. Levels of microRNA are changing constantly and with the current knowledge on the topic it is impossible to predict a risk of developing specific diseases in the future. Cryotubes not used to microRNA/exosome analysis will be stored as part of a biobank.

Extracellular vesicle analysis:

Extracellular vesicles (EVs, also known as exosomes) will be isolated from plasma samples before characterization of surface markers and content. EVs are isolated by a number of different techniques, ultra centrifugation, precipitation, size exclusion chromatography among others. While protein characterization will be done using classical molecular biological techniques such as ELISA and Western blots in addition to array techniques, EVarray that utilises a panel of antibodies directed against known EV surface markers. These analyses might be supported by proteomic analysis of all proteins as well as post-translational modifications such as phosphorylations and glycosylations.

In addition, the nucleic acid (DNA and RNA including miRNA) content of EVs will be analysed using next generation sequencing (NGS), qRT-PCR, and other nucleic acid detection techniques. Both the protein data and the nucleic acid data will be subjected to bioinformatic analysis using different pipelines and analysis tools depending on the dataset and the purpose of the analysis. A number of validated published databases will be used for annotation and comparison. EVs do not contain genomic DNA and the analysis are therefore not a mapping of the subjects genome. As EV secretion and nucleic acid content in these are thought to change rapidly in the body due to external and internal signals and are considered a snapshot at the specific time of blood sampling. With the current knowledge on the topic it is impossible to predict a risk of developing specific diseases in the future.

Coagulation assays:

Functional and immunologic plasma assays will be employed to analyze proteins and pathways in coagulation and fibrinolysis. The analyses will be performed in the accredited clinical laboratory and the thrombosis and hemostasis research unit at the Department of Clinical Biochemistry, Aarhus University Hospital. Plasma samples will be stored in cryotubes at -80 °C until they will be analyzed in large batches.

Ektacytometry for Erythrocytic Deformability:

The rheological properties of RBCs are quantified using a Deformability or Elongation Index (DI or EI). A higher EI at the optimum viscosity (300 Osmolality) indicates highly deformable RBCs indicative of better microcirculation, while a lower EI indicates rigid and fragile RBCs resulting into impaired microcirculation and tissue hypoxia. Briefly, 6-uL of heparinized fresh blood is mixed with 600-uL of PVP solution (300 Osm)

and transferred to a disposable kit (K01 Deformability Test Kit; RheoMeditech, South Korea). The kit is placed inside the laser-assisted ektacytometer (RheoScan AnD300, RheoMeditech, Seoul, S. Korea) for automated read out, data and image collection as per the vendor's instructions.

Analytical Flow Cytometry (FC) for eryNOS3 phosphorylation (pNOS3Ser1177) and s-nitrosylation (-SNO) in RBCs:

Red blood cells are separated from 50- μ L of freshly fixed blood samples, using a cocktail of monoclonal antibodies (mAb) to RBC-specific markers (Glycophorin A, GPA; and Hemoglobin, Hb; Bioss Biotech, USA). To assess the functional features, after fixation and permeabilization, RBCs are incubated with antibodies conjugated to fluorochrome either directly or through secondary antibodies to s-nitrosocysteine (-SNO; Abcam), and pNOS3Ser1177 (Bioss). Next, RBC samples are run through a flow cytometer (FACS Calibur, BD Biosciences, San Diego, CA), and data is collected using CellQuest software to process for FC analysis.

Prestroke physical activity measures in AIS and ICH patients

(Substudy only at Aarhus University Hospital)

Patient and their relatives will be asked to complete the physical activity in the elderly (PASE) questionnaire during the acute hospital admission. In patients who are unable to complete it themselves an structured interview based on the PASE questionnaire will be performed^{41,42}

The PASE is a 12-item questionnaire, which quantifies the amount of PA over a 7-day period. The PASE questionnaire was developed with the purpose of assessing the level of PA in middle-aged and elderly individuals. The PASE score is calculated by taking the average number of hours spent on an activity (sports, occupational activity, household activities, and leisure time activities) per day over a 7-day period multiplied by an activity coefficient. Item scores are added to reveal the PASE score. The PASE score may range from zero to more than 400.

9. Benefit of the study

Potential benefits:

Participating patients with AIS and ICH may experience an immediate reduction of their neurological symptoms and a persistent reduction of disability at long-term follow-up.

Disadvantage:

Mild-to-moderate pain and petechiae in the RIC-treated arm may occur during the inflation of the blood pressure cuff. Otherwise, the RIC treatment has been proven safe and without side effects.

Sham-RIC will only be associated with a slight sensation of pressure on the upper extremity.

Blood samples (only patients transported to and admitted at Aarhus University Hospital) of approximately \approx 57mL (19mL prehospital and 2x19 \pm mL in-hospital) will be drawn.

An additional (compared to standard operating procedure) head CT will be performed at 7 days in ICH patients included at Aarhus University Hospital (If possible, the scan will be performed at the hospital were the patient is admitted at day 7). The estimated added whole-body radiation dose of the 7 days CT-scan is

on average 1,5 mSv (range 1,05 to 1,95) corresponding to 6 months of accumulated background radiation in Denmark (Danish Health Authority). This corresponds to an estimated increased life-time cancer risk of 0.005%.

10. Assessment of safety

Emergency unblinding procedure

All on call neurologists will have access to electronic case report form using a personal and password protected login to perform randomization of study participants. In cases where emergency unblinding is necessary the on call neurologist will logon to electronic CRF at ddsc.dk and enter the civil registration number (CPR) of the patient. The treatment can now be unblinded. All changes will leave an audit-trail.

Adverse events

Patients are monitored in the stroke unit with NIHSS scoring and Scandinavian Stroke Scale (SSS) at close intervals, and adverse events are treated according to clinical guidelines.

The Data Monitoring Committee will assess safety according to primary study endpoints, recurrent stroke, myocardial infarction and mortality.

Interim analysis of safety parameters (hematoma expansion at 24 hours and mortality) in ICH will be performed regularly during the study.

During the acute in-hospital phase the patients will be asked if they have experienced any deterioration of health or new symptoms during or after treatment with the investigational device. Furthermore all patients will have a 24/7 contact number to the stroke center and a contact to study research physician and will be instructed to report any deterioration of health or new symptoms. All discharged patients (and relatives) will be given contact details to study personnel and be instructed to report any deterioration of health or new symptoms. The patients with acute ischemic stroke and intracerebral hemorrhage will once again be asked for adverse events at the 3 month telephone interview. In this group, end-of-study visit is the last telephone interview, whereas end-of-study visit for participants without a vascular diagnosis or TIA(without DWI lesion) is at the time of discharge from the stroke center.

All patients, who have consented, are followed (through Danish Health Registries, LPR and DSR) for new vascular events and mortality for up to 12 months.

Adverse events and adverse device events are defined according to ISO 14155:2011 and European Commission guideline on medical devices (MEDDEV 2.7/3 revision 3).

Adverse event (AE):

Any untoward medical occurrence, unintended disease or injury or any untoward clinical signs (including an abnormal laboratory finding) in subjects, users or other persons whether or not related to the investigational medical device.

Serious Adverse Event (SAE)

Adverse event that:

- a) led to a death, injury or permanent impairment to a body structure or a body function.
- b) led to a serious deterioration in health of the subject, that either resulted in: - a life-threatening illness or injury, or - a permanent impairment of a body structure or a body function, or - in-patient hospitalization or prolongation of existing hospitalization, or - in medical or surgical intervention to prevent life threatening illness

Device Deficiency

Inadequacy of an investigational medical device related to its identity, quality, durability, reliability, safety or performance. This could be malfunctions, use error, or inadequacy in the information supplied by the manufacturer.

Adverse Device Effect

Adverse event related to the use of an investigational medical device.

This includes any adverse event resulting from insufficiencies or inadequacies in the instructions for use, the deployment, the implantation, the installation, the operation, or any malfunction of the investigational medical device.

This includes any event that is a result of a use error or intentional abnormal use of the investigational medical device.

Serious Adverse Device Effect (SADE)

Adverse device effect that has resulted in any of the consequences characteristic of a serious adverse event.

Unanticipated Serious Adverse Device Effect (USADE)

Serious adverse device effect which by its nature, incidence, severity or outcome has not been identified in the current version of the risk analysis report.

Anticipated SADE (ASADE): an effect which by its nature, incidence, severity or outcome has been previously identified in the risk analysis report

Event report and causality assessment

The relationship between the use of the medical device and the occurrence of each adverse event will be assessed and categorized. All events will be registered in the electronic CRF and reported to the authorities at the interim analysis. A yearly safety report, containing a list of all SAEs/SADEs and near-miss incidents, thorough evaluation of individual events, risk/benefit analysis of the investigational medical device and safety conclusions, will be submitted to the Danish Health and Medicines Authority and the Independent Ethics Committee by the Sponsor as required by Danish law.

Exceptions to and special considerations about reporting procedures are stated below:

Acute stroke is an acute life threatening disease with a high risk of neurological deterioration and mortality. The natural history of stroke is associated with a high risk of complications (stroke in progression, hemorrhagic transformation, dysphagia, cardiac arrhythmia, pneumonia, and other serious infections). These *complications* all relates to the index stroke (occurring *before* randomization) and is foreseeable. If an event occurs that based on the assessment by the investigator is a result of the natural history of the

disease it will not be reported by the sponsor to the Danish medicines agency and regional ethics committee within 7 days/2 days (see list of foreseeable events/complications below).

All expected serious adverse events will be reported to the Danish Medicines Agency (and regional ethics committee) every 3 months.

All *new* serious events (Serious Adverse Events), complications that are not foreseeable or device near incidents or malpractice will be reported by the sponsor to the authorities (within 7 days, or 2 days if there is a risk of event reoccurrence) according to ISO 14155:2011 and European Commission guideline on medical devices (MEDDEV 2.7/3 revision 3). Investigators will report the event to sponsor (within 24 hours). Sponsor will as fast as possible and no later than 7 days, or 2 days if there is a risk of event reoccurrence, report the event to the Danish Medicines Agency and regional ethics committee.

All Serious Adverse Device Effect (SADE) will be reported to the Danish Medicines Agency (and regional ethics committee) within 7 days, or 2 days if there is a risk of event reoccurrence

List of foreseeable adverse events/complications

Foreseeable events/complications that will not be reported by the sponsor to the Danish medicines agency and regional ethics committee within 7 days/2days.

| Patient Population | Event | Frequency | Mitigation/treatment |
|--------------------|--|-----------------------------|---|
| TIA and AIS | Recurrent stroke. | 10% at 90 days ^a | Standard medical treatment, intravenous thrombolysis/mechanical thrombectomy according to patient characteristics |
| AIS | Symptomatic intracerebral hemorrhage (related to IV tPA or EVT) | 7% ^b | Antithrombotic/anticoagulation therapy cessation. Antifibrinolytic agent. Bloodpressure control. |
| AIS, ICH | Early (<24hours) and late (>24hours) neurological deterioration (Δ NIHSS \geq 2 or death) | 7-22% ^c | According to etiology |
| AIS, ICH | Aspiration pneumonia | 5-20% ^d | Antibiotic treatment according to hospital SOP |
| AIS, ICH | Urinary tract infections | 12% ^e | Antibiotic treatment according to local hospital SOP |
| AIS, ICH | Severe infections (sepsis) | 13% ^f | Antibiotic treatment according to local hospital SOP |
| AIS, ICH | Cardiac arrhythmia | 29% ^g | Depending on arrhythmia type. Treatment according to cardio.dk |

| | | | |
|-----------------|--|---|--|
| AIS, ICH | Acute myocardial infarction | 6% ^h | Treatment according to cardio.dk and conference call with cardiologist |
| AIS, ICH | Deep venous Thrombosis(DVT)/pulmonary embolism(PE) | DVT: 8% ⁱ PE:1% ^j | Treatment according to cardio.dk |
| AIS, ICH | Falls (in-hospital phase) | 14-65% ^{k,l} | Treatment according to type of injury |
| AIS, ICH | Seizure | AIS:3-10% ^m ICH:8% ⁿ | Treatment according to local SOP |
| AIS/ICH | Mortality 30 days | AIS:10-20% ^m ICH:30% ⁿ | |

^{a)} Wang Y et al. Clopidogrel with Aspirin in Acute Minor Stroke or Transient Ischemic Attack. NEJM. 2013; 369:11-19

^{b)} Berkhemer OA. A Randomized Trial of Intraarterial Treatment for Acute Ischemic Stroke. NEJM. 2015; 372:11-20

^{c)} Kwan J et al. Early neurological deterioration in acute stroke: clinical characteristics and impact on outcome Journal of the Association of Physicians, 2006, Sept; 99:9: 625-633

^{d)} Hannawi Y et al. Stroke-Associated Pneumonia: Major Advances and Obstacles. Cerebrovasc Dis. 2013;35:430-443

^{e)} Bogason E. Urinary Tract Infections in Hospitalized Ischemic Stroke Patients: Source and Impact on Outcome. 2017;9(2):e1014. doi:10.7759/cureus.1014

^{f)} Berger B. Epidemiologic features, risk factors, and outcome of sepsis in stroke patients treated on a neurologic intensive care unit. Journal of Critical care. 2013;29(2)

^{g)} Daniele O et al. Stroke and cardiac arrhythmias. J Stroke Cerebrovasc Dis. 2002 jan-feb;11(1):28-33

^{h)} Johnston KC et al. Medical and neurological complications of ischemic stroke: experience from the RANTTAS trial. RANTTAS Investigators. Stroke. 1998;29(2):447

ⁱ⁾ Bembenek J. Early stroke-related deep venous thrombosis: risk factors and influence on outcome. J thromb Thrombolysis. 2011 Jul; 32(1):96-102

^{j)} Pongmoragot J. et al. Pulmonary Embolism in Ischemic Stroke: Clinical Presentation, Risk Factors, and Outcome. J Am Heart Ass. 2013 Dec;2(6)

^{k)} Davenport RJ, Dennis MS, Wellwood I, Warlow CP. Complications after acute stroke. Stroke. 1996; 27: 415–420.

^{l)} Teasell R, McRae M, Foley N, Bhardwaj A. The incidence and consequences of falls in stroke patients during inpatient rehabilitation: Factors associated with high risk. Arch Phys Med Rehabil. 2002; 83: 329–333.

^{m)} Bladin C, Alexandrov AV, Bellavance A et al: Seizures After Stroke: A Prospective Multicenter Study. Arch Neurol. 2000;57(11):1617-1622.

ⁿ⁾ Woo K-M, Yan S-Y, Cho K-T. Seizures after Spontaneous Intracerebral Hemorrhage. J. Korean Neurosurg Soc. 2012 Oct;52(4)312-319

^{o)} Schwartz J et al. Incorporating Stroke Severity Into Hospital Measures of 30-Day Mortality After Ischemic Stroke Hospitalization. Stroke. 2017. Ahead of print: <https://doi.org/10.1161/STROKEAHA.117.017960>

^{p)} Lichtman JH et al. 30-Day Mortality and Readmission after Hemorrhagic Stroke among Medicare

List of anticipated adverse device effects

| RIC device | Frequency | Mitigation/treatment |
|------------------------|-------------------|--|
| Local petechiae | 4-5% ^a | none |
| Discomfort/pain | 20-30% | Treatment stopped if the non-competent patient verbally or non-verbally demonstrates intolerable discomfort or if the competent patient verbally want to discontinue the treatment |

| | | |
|--|------------------------------|---|
| Near events (device related) | None known in the literature | |
| Acute limb ischemia (upper extremity) – theoretical complication. | None known in the literature | Treatment stopped. Treatment according local hospital SOP's for acute limb ischemia. Known atherosclerotic stenosis in upper extremities are <i>an exclusion criteria.</i> See Investigators Brochure for further details. |
| Sham RIC device | | |
| None known | | |

a) Hausenloy D et al. Remote Ischemic Preconditioning and Outcomes of Cardiac Surgery. New England J. Medicine. 2015 Oct;373(15):1408-17

Treatment and mitigation of any adverse events will be according regional guidelines.

Adverse events as a result of standard treatment will be handled according to local guidelines. Adverse events may also occur as a suspected reaction to RIC/Sham RIC – in these cases, the conditioning stimulus will be stopped immediately and appropriate treatment will be initiated.

In cases of SAE and device related near events all patients will be followed until resolution of symptoms/signs and/or all clinical relevant treatment have been performed and/or until the symptoms/signs are in a stable phase.

11. Project timetable and recruitment feasibility

Study preparation: January to February 2017

Study start date: March 2018

Month 0-60: Patient recruitment

Expected inclusion end date: 31. December 2022

Month 60-66: Study analysis

Expected end date for last analysis and follow-up: 31. December 2024

Patient inclusion per center:

Aarhus University Hospital: 50%

Regional Hospital West Jutland (Holstebro): 12,5%

Odense University Hospital: 12,5%

Aalborg University Hospital: 12,5%

Zealand University Hospital (Roskilde): 12,5%

12. Ethical considerations

The study will await approval from the Regional Ethical Committee, the Danish Health Authority, and the Danish Data Protection Agency. Enrollment of additional Danish stroke centers will require a new application to the Danish Medicines Agency and the regional ethics committee (amendment).

A large acute stroke results in immediate, major neurological deficits (+/-aphasia) and a substantially increased risk of a long-term reduction of functional capability. This group is assessed as legally incompetent. Even in patients suffering from minor stroke, acute cognitive impairment are common, making individual preference on acute treatment options impossible. In order to preserve brain tissue, the intervention must be applied without delay. Improvement of standard care in patients suffering from acute stroke is of paramount importance. Inclusion of all stroke patients is necessary in order to translate any positive research results into a benefit for all stroke patients. The intervention is without any known risk apart from moderate pain in the upper extremity when the cuff is inflated. We find that the conditions for an acute study are satisfied since the majority of the patients are incapacitated at stroke onset and the treatment has to be initiated as soon as possible and there are no reported serious side effects from RIC.

Study participants are covered in accordance with the Danish Patient Insurance Act.

13. Data handling and record keeping

All study data are recorded in an electronic CRF with blinded data and identification via a study identification number. The study will apply to the specifications of act on processing of personal data.

The database(e-CRF) is handled by OPUS consult Aps, Egaa, Denmark, who already handles the Danish Stroke Center Database in Aarhus (ddsc.dk)

Remote Ischemic Conditioning in Stroke (RISC) is a predefined collaboration in which three large RIC trials have agreed to share anonymized patient data for a large metaanalysis once the individual trials have ended (Principle Investigators of the individual trials are: Grethe Andersen (DK), Philip Bath (UK) and Fernando Pico (France).

Patient participation will be recorded in the medical record. Data will be stored at the Department of Neurology, Aarhus University Hospital, for 15 years, after which the documents will be shredded.

A notification to The Danish Data Protection agency will be submitted.

During the entire study period and at all participating centers the local GCP unit will perform quality assurance control including source data verification.

A complete list of all source data will be made and approved by the local GCP unit before study initiation.

The investigator permits direct access to all source data/documents (including electronic patient record) at monitoring visits, audits and/or inspections by the regional ethics committee and Danish Medicines Agency.

14. Publications policy

The results of the study, both negative, inconclusive and positive, will be disseminated as widely as possible - through publication in an international peer-reviewed journal, as conference presentations and on www.clinicaltrials.gov.

The trial is registered on ClinicalTrials.gov with Identifier: NCT03481777

15. Statistics

Primary study endpoint:

Difference in clinical outcome (mRS) at 3 months in acute ischemic stroke (*General ordinal logistic regression analysis. Significance level of 5%*)

The **main analysis of the primary study endpoint** will be performed using the entire range of the modified Rankin Scale (**General ordinal logistic regression, unadjusted**) on the population fulfilling the **in-hospital inclusion criteria (target population)**. The trial success analysis is performed on the **target population** which consists of prehospital randomized patients with an in-hospital diagnosis of acute ischemic stroke (including TIA with a DWI lesion) and intracerebral hemorrhage (ICH) regardless of adherence to investigational treatment (**modified Intention-To-Treat analysis**).

Supplementary safety analysis:

All patients randomized in RESIST had pre-randomization a focal neurological deficit (documented on the prehospital stroke score). The presence or absence of specific focal neurological symptoms are assessed again after 24 hours using the same score as pre-randomization (PreSS) in all randomized patients (PreSS 24 hour/ at discharge). The primary safety analysis will be made on the **entire randomized population using the difference in prehospital stroke score (PreSS)** to document change in neurological deficits between pre-randomization and 24 hour/discharge (**General ordinal logistic regression, unadjusted**). There has been no safety concern over RIC treatment in patients with non-vascular diagnosis^{23,27,28,29,30}

Primary endpoint

| | |
|---|--|
| <p style="text-align: center;">Acute Stroke <i>AIS (incl TIA+DWI) and ICH</i> <i>Modified Intention-To-Treat</i></p> | <p style="text-align: center;"><u>Entire range of 3 months</u> <u>modified Rankin Scale</u> General ordinal logistic regression, unadjusted</p> |
|---|--|

Supplementary safety analysis

| | |
|--|--|
| <p style="text-align: center;">All prehospital randomized patients (AIS, TIA+DWI, ICH, non-stroke)</p> | <p style="text-align: center;"><u>Difference in <i>PreSS</i></u> (PreSS_{prehospital} to PreSS_{24h/discharge}) General ordinal logistic regression, unadjusted</p> |
|--|--|

If remote ischemic preconditioning is associated with ultra-early treatment effect and reduced symptom severity (in the ambulances), this could affect the in-hospital indication for IV-tPA and/or EVT (in the case of rapid improvement of symptoms or mild symptoms)

Because of this, a **supplementary analyses** using **general ordinal logistic regression, adjusted for a possible** imbalances in reperfusion treatments (IV-tPA and EVT) between treatment arms will be made.

Secondary study endpoints (selected):

- Difference in prehospital stroke score (PreSS) (Supplementary safety endpoint, see above) (All randomized patients, general ordinal logistic regression, significance level 5%)
- Difference in prehospital stroke score (PreSS) during the first 24 hours: (general ordinal logistic regression analysis in the stroke subtypes, Significance level of 5%)
- Difference in early neurological improvement in AIS and ICH (*general ordinal logistic regression analysis. Significance level of 5%*)
- Difference in prehospital stroke score (PreSS) after 24 hours in subgroups: IV tPA/EVT treated AIS and ICH. (ordinal logistic regression analysis, significance level of 5%)
- Difference in acute hematoma expansion in patients with ICH (Binomial regression analysis, significance level of 5%)
- Difference in acute infarct growth in patients with AIS treated with IV-tPA and/or EVT (Binomial regression analysis, significance level of 5%)
- Major adverse cardiac and cerebral events (MACCE) and recurrent ischemic events at 3 and 12 months in AIS patients (*Cox regression analysis, significance level of 5%*)
- Three-month and one-year mortality (*Two sample test of proportion (Chi-square test), significance level of 5%*)

- EVT-eligibility (MRI assessed) in RIC treated AIS patients with large vessel occlusion (LVO) (*Binomial regression analysis. Significance level of 5%*)

Sample Size

The treatment effect of RIC on long-term functional outcome is unknown. We have assumed a small but clinical significant neuroprotective effect corresponding to a 6-7% increased odds for a beneficial shift on the modified Rankin Scale. The sample size calculation was based on a simulation-based approach to the analysis of statistical power when ordinal logistic regression analysis is performed (significance level of 5%)

The statistical power was simulated at different hypothetical sample sizes (on the target population) (ranging from 200 to 1900) with 2000 simulation-runs performed at each step. Unpublished data on IV-tPA and/or EVT treated AIS patients and ICH patients from our institution were used:

3 months modified Rankin Scale distribution (proportions) in 2017 for patients with ICH or IV tPA/EVT treated AIS at Aarhus University Hospital.

| Modified Rankin Scale | mRS 0 | mRS 1 | mRS 2 | mRS 3 | mRS 4 | mRS 5 | mRS 6 |
|-----------------------|-------|-------|-------|-------|-------|-------|-------|
| Proportion | 0.139 | 0.273 | 0.141 | 0.110 | 0.145 | 0.07 | 0.126 |

Based on our previous trial experience with prehospital remote ischemic conditioning we estimate that a sample of 1000 subjects with target diagnosis (AIS and ICH) will be feasible to include during the study period.

Thus, including 1000 patients with target diagnosis provide sufficient power at a significance level of 5% to detect RIC treatment effects of the estimated 6-7% (*see table below*)

| Treatment effect (assumed neuroprotective) | 5% | <u>6%</u> | <u>7%</u> |
|--|------|-------------|-------------|
| Sample (target diagnosis), <i>n</i> | 1000 | <u>1000</u> | <u>1000</u> |
| Power | 66% | <u>80%</u> | <u>90%</u> |
| Alfa level (significance level) | 5% | <u>5%</u> | <u>5%</u> |

The estimated prehospital, randomized, sample:

| Sample size, prehospital | Proportion of randomized | <i>n</i> = |
|--|--------------------------|------------|
| Target diagnosis (AIS and ICH), <i>n</i> | 67% | 1000 |
| Non-vascular diagnosis | 27% | 403 |
| TIA without DWI lesion | 4% | 60 |
| Lost to follow-up | 2% | 30 |
| Total | | 1492 |

| | | |
|------------------------|--|-------------|
| Plan to include | | 1500 |
|------------------------|--|-------------|

We therefor plan to include 1500 patients with a prehospital putative stroke in order to get 1000 patients with the target diagnosis of acute ischemic stroke and intracerebral hemorrhage. There is no planned replacement of patients lost to follow-up.

Interim analysis

We will perform an interim analysis for evaluation of the actual event rate after inclusion of 50% of the patients. If this shows a lower good outcome rate than expected, a new sample size calculation will be performed. Furthermore, the Data monitoring committee (DMC) will perform an independent safety analysis and will review the overall safety and efficacy according to the DMC charter (planned in the early phase of the trial, at 12 month and hereafter at least every 18. months) An additional safety interim analysis of mortality and hematoma expansion in ICH patients will be performed. Interim analysis are assessed using a level of significance of 1%.

The trial can be stopped at interim analyses for both futility, efficacy and safety reasons.

Detailed description is found in the DMC charter.

Missing data:

Missing data will be handled using multiple imputation (predictive mean matching imputation).

Deviations from the statistical plan:

All deviations from the statistical plan will first be applied when approved by the regional ethics committee and the Danish Medicines Agency.

Trial decision making and stopping rules

A Data Monitoring Committee (DMC) will be appointed before trial start and include a signed DMC charter. The DMC will review the accumulated data during RESIST trial and provide advice on the conduct of the trial to the Steering Committee.

The DMC should inform the Steering Committee if, in their view:

1. the results are likely to provide convincing evidence that one trial arm is clearly indicated or contraindicated, and there is a reasonable expectation that this new evidence would materially influence patient management
- or
2. it is beyond doubt that no clear outcome would be obtained (futility)

It is not expected that RESIST trial will be stopped for reasons of efficacy or futility based on observed treatment differences. A predefined pooling of individual patient data (Remote Ischemic conditioning in Stroke Collaboration, RISC) is planned. To dispel scepticism, a robust demonstration of efficacy of this simple intervention is required. The primary reason to recommend stopping the trial will be for safety reasons.

For Acute Ischemic Stroke and Intracerebral Hemorrhage:

- For the primary endpoint the DMC will consider stopping if there is a robust improvement in functional outcome after 3 months (modified Rankin Scale) for Acute Ischemic Stroke and Intracerebral haemorrhage in the sham (control) group compared to the intervention (RIC) group, achieving $p < 0.01$.
- A substantial number of patients experience serious adverse events ($p < 0.01$)
- DMC will consider stopping if there is an increase in mortality or stroke recurrence for Acute Ischemic Stroke and Intracerebral Hemorrhage patients in the intervention (RIC) group compared to the control (sham) group, achieving $p < 0.01$.
- For ICH the DMC will consider stopping/modification if there is a clear evidence of hematoma growth during the first 24-hour in the intervention group, achieving $p < 0.01$

The DMC will make recommendations, which could include:

- No action needed, trial continues as planned
- Early stopping due, for example, to clear benefit or harm of a treatment, futility, or external evidence
- Stopping recruitment within a subgroup
- Extending recruitment (based on actual control arm response rates being different to predicted other than on emerging differences) or extending follow-up
- Approving and/or proposing protocol changes

16. Source data access and monitoring

Trial-related audits and/or monitoring will be provided by direct access to source data/documents. Local monitors from the Unit for Good Clinical Practice, Aarhus University, will perform the audit and monitoring. Audit and monitoring will, likewise, be performed by qualified local GCP-monitors when enrollment at other stroke centers in Denmark is starting. Before enrollment can start a trial monitoring plan must be available. The audit and monitoring process will involve a 100% monitoring of signed consent forms (“samtykkeerklæringer og fuldmagtserklæringer”) and serious adverse events.

17. Economy

Study initiator is Grethe Andersen, Senior Consultant, MD, DMSc, Professor of Neurology at Department of Neurology, Aarhus University Hospital.

The Danish foundation Vilhelm Petersens mindelegat has supported the study with 1.240.000 dkr (research nurse salary and “stroke RIC” devices).

The Danish foundation TrygFonden has supported the study with 4.000.000 dkr (research personnel salaries, running costs, micro RNA analysis and investigational devices).

Aarhus University has supported the study with 550.000 dkr (study investigator/coordinator salary)

The funders have no role in study design, data collection, analysis or interpretation, nor the decision to publish or the preparation, review and approval of the manuscript.

Stroke RIC/Sham-RIC device is developed in collaboration with Aarhus University, Faculty of Biomedical Engineering, 8200-DK, Aarhus N, Denmark; Seagull Aps, 4160-DK, Herlufmagle, Denmark and the Department of Neurology, Aarhus University Hospital, 8000-DK, Aarhus C, Denmark. Seagull Aps has no role in study design, data collection, analysis or interpretation, nor the decision to publish or the preparation, review and approval of the manuscript.

The research grants are transferred directly to a research account administered by the financial department of Aarhus University Hospital. None of the involved doctors or research nurses has any conflict of interest or economic advantages in regards to the study. There is no economic compensation or reimbursement for patients participating in the study.

Supplements

Supplement A - Neuroimaging protocol

On admission, either CT or MRI is performed according to hospital SOP.

All AIS and ICH patients can be included in the in-hospital RIC/sham-RIC based on a baseline native CT. However, patients with complete remission of symptoms after randomization (TIA) require an MRI at baseline demonstrating acute ischemic lesion on DWI in order to continue the randomized treatment. TIA patients with a documented ischemic lesion are, per definition, handled as ischemic stroke in this study. Baseline MRI in TIA patients is performed before the next RIC series (MRI <6 hours from RIC/sham-RIC last cuff). Patients with AIS who are not eligible for IV tPA due to contraindications will have CT/MRI before their next RIC/sham-RIC series (< 6 hours from RPerC last cuff) according to hospital SOP. All ICH patients will have a baseline and 24-hour CT (hospital SOP)

Non-stroke diagnosis and TIA (only evaluated with CT or TIA without DWI lesion on MRI) will not be included in the in-hospital RIC study and will receive no further RIC treatment or neuroimaging.

| Imaging protocol | Accepted time frame |
|---|---|
| MRI baseline – AIS IV tPA eligible | Acute on admission |
| CT baseline – AIS IV tPA eligible | Acute on admission |
| MRI/CT baseline – AIS non IV tPA eligible | < 6 hours from last RIC cuff |
| MRI (24-hour) - tPA/EVT treated AIS patients* | 18-30 hour from baseline MRI |
| MRI baseline – ICH | Perform CT as soon as possible and < 3 hours from last RIC cuff |
| CT baseline – ICH | < 6 hours from last RIC cuff |
| Control CT (24-hour) – ICH* | 18-30 hour from baseline CT |
| Control CT (7-day)* | 5-9 days from baseline CT |

*Substudy at Aarhus University Hospital

Computed tomography (CT) protocol

Baseline CT and CT angiography are obtained in centers using CT for primary assessment (according to hospital SOP).

CT analysis

Hematoma volume assessment at baseline and at 24 hours and 7 days is performed by an experienced neuroradiologist. The assessment will be assisted using an automated stroke volume assessment software (e.g. COMBAT stroke APS, Aarhus University Hospital, Denmark) or using the formula $ABC/2$ where A is the greatest hematoma diameter by CT, B is the diameter perpendicular to A , and C is the number of CT slices with hematoma multiplied by slice thickness⁴³.

Magnetic resonance imaging (MRI) protocol

Diffusion-weighted imaging (DWI), apparent diffusion coefficient (ADC), T2* gradient-recalled echo (T2*GRE), and T2 fluid-attenuated inverse recovery (T2-FLAIR). MR angiography time-of-flight (MRA-TOF) is performed according to hospital standard operating procedures (SOP).

The 24-hour MRI is performed on the same MRI scanner used at baseline, if possible. This protocol includes: DWI, T2*, T2 and T2-FLAIR. MRA-TOF at 24 hours is obtained if the baseline CT-angiography or MRA-TOF demonstrated vessel occlusion.

The total acquisition time for this protocol is approximately 7-10 mins (MRI)/10-15 min (MRI + MRA-TOF), depending on the MRI equipment.

MRI analysis

Acute (baseline) and 24-hour follow-up (DWI lesion will be outlined, representing irreversibly damaged tissue (*substudy at Aarhus University Hospital*)).

Infarct growth is defined as the difference between baseline DWI and the 24-hour MRI-DWI lesion.

Infarct volume assessment at baseline and at 24 hours is performed by an experienced neuroradiologist. The assessment will be assisted using an automated stroke volume assessment software (e.g. COMBAT stroke APS, Aarhus University Hospital, Denmark) or using the formula $ABC/2$ where A is the greatest hematoma diameter by CT, B is the diameter perpendicular to A , and C is the number of CT slices with hematoma multiplied by slice thickness⁴³

Vascular patency and reperfusion are assessed using the Thrombolysis in Cerebral Infarction Perfusion Scale (TICI) criteria⁴⁴

- No perfusion (TICI 0)
- Perfusion past the initial occlusion, but no distal branch filling (TICI 1)
- Perfusion with incomplete or slow distal branch filling (TICI 2)
- Full perfusion with filling of all distal branches, including M3 and M4 (TICI 3)

Supplement B – Baseline data

| | |
|---|--|
| Age | Platelet inhibitor treatment |
| Male/female | Anticoagulation therapy |
| Medical History | New oral anticoagulation treatment (NOAC) |
| Hypertension | Opioid treatment |
| Smoking | SSRI treatment |
| Alcohol | Clinical and physiological data |
| Hyperlipidemia | Prehospital Stroke Score (PreSS) |
| Diabetes | Modified Rankin Scale prestroke |
| Previous myocardial infarction <i>Yes: within 3 months, yes: more than 3 months, no, unknown</i> | NIHSS (baseline) |
| Angina pectoris | Symptom onset (time) |
| Recent angina pectoris (< 4 weeks) <i>If yes, date for latest episode</i> | Time of admission |
| Atrial fibrillation | Completed prehospital RIC/sham cycles |
| Previous ischemic stroke | = 5, 4, 3, 1-2, 0 |
| Stroke < 3 month <i>If yes, date</i> | Time (start/stop) for each RIC/sham cycle |
| Previous TIA | NIHSS (t = 24 hours) |
| Recent TIA < 4 weeks <i>If yes, date for latest episode</i> | Stroke etiology (TOAST) |
| Previous ICH | Admission blood pressure |
| Peripheral artery disease | IV tPA/EVT treated AIS |
| Physical activity (PASE interview) <small>Substudy at Aarhus University Hospital</small> | Treatment initiation (time) |
| Medication on admission | Quality indicators (Danish Stroke Registry) |
| Statins | WHO-5 <small>Substudy at Aarhus University</small> |
| Calcium channel blockers | |
| ACE inhibitors/angiotensin receptor blockers | |
| Beta-blockers | |

Baseline data obtained from treating physician, database of Danish stroke center (ddsc.dk), Danish stroke registry and electronic health records.

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